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Progression of Chronic Liver Inflammation and Fibrosis Driven by Activation of c-JUN Signaling in *Sirt6* Mutant Mice*[S]

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Background: Sirt6 plays important roles in metabolism and lifespan; however, its role in inflammation is unknown.
Results: Sirt6 deficiency in the immune cells of mice results in liver inflammation and fibrosis through activating the c-JUN signaling.
Conclusion: Sirt6 has anti-inflammatory function in mice.
Significance: Small chemical compounds that activate Sirt6 might be useful in therapeutic treatment of chronic liver inflammation.

The human body has a remarkable ability to regulate inflammation, a biophysical response triggered by virus infection and tissue damage. Sirt6 is critical for metabolism and lifespan; however, its role in inflammation is unknown. Here we show that Sirt6-null (*Sirt6*$^{-/-}$) mice developed chronic liver inflammation starting at ~2 months of age, and all animals were affected by 7–8 months of age. Deletion of Sirt6 in T cells or myeloid-derived cells was sufficient to induce liver inflammation and fibrosis, albeit to a lesser degree than that in the global Sirt6$^{-/-}$ mice, suggesting that Sirt6 deficiency in the immune cells is the cause. Consistently, macrophages derived from the bone marrow of *Sirt6*$^{-/-}$ mice showed increased MCP-1, IL-6, and TNFα expression levels and were hypersensitive to LPS stimulation. Mechanistically, SIRT6 interacts with c-JUN and deacetylates histone H3 lysine 9 (H3K9) at the promoter of proinflammatory genes whose expression involves the c-JUN signaling pathway. Sirt6-deficient macrophages displayed hyperacetylation of H3K9 and increased occupancy of c-JUN in the promoter of these genes, leading to their elevated expression. These data suggest that Sirt6 plays an anti-inflammatory role in mice by inhibiting c-JUN-dependent expression of proinflammatory genes.

Inflammation is a complex biophysical response of the body to pathogen infection and tissue injury. Immune cells are activated during inflammation and are recruited to the site of damage to initiate the healing process by eliminating pathogens and damaged cells (1–4). Although acute inflammation is considered protective, chronic inflammation is associated with many diseases (5–8). Much evidence indicates that inflammation is tightly regulated by many factors, including cytokines, signaling molecules, and transcription factors, such as TGF-β, activator protein-1 (AP-1),[3] TNF-α, NF-κB, sirtuin-1 (SIRT1), and STATs, all of which affect or regulate expression of cytokines (1–4).

Both SIRT1 and SIRT6 belong to a family consisting of seven sirtuins (SIRT1–7) that share homology with yeast Sir2. These proteins localize to different compartment of the nucleus (SIRT1, SIRT6, and SIRT7), the mitochondrion (SIRT3, SIRT4, and SIRT5), and the cytoplasm/nucleus (SIRT2) (9, 10). Sirtuins serve as NAD^+ dependent type III histone deacetylases and also deacetylate many proteins that play important roles in numerous biological processes (11–14). Currently, all seven sirtuins (Sirt1–7) have been mutated in mice by gene targeting, and the mutant mice exhibited distinct phenotypes (15–25). *Sirt1*-null mice exhibited the most severe phenotype and died at middle gestation to perinatal stages (23, 24). Using a tissue-specific *Sirt1* knock-out mouse model to bypass early lethality, it was shown that deletion of *Sirt1* in macrophages activates NF-κB activity, resulting in increased transcription of several proinflammatory target genes (26). *Sirt1* was also reported to suppress transcriptional activity of AP-1 and expression of a proinflammatory gene cyclooxygenase-2 in macrophages (27).

It was previously reported that a strain of *Sirt6*-deficient mice carrying a targeted disruption of exon 1 died at ~4 weeks of age when they were on a 129 genetic background. These mice displayed profound lymphopenia, loss of subcutaneous fat, lordokyphosis, low insulin and hypoglycemia, and premature aging (17). Our analysis of *Sirt6*$^{-/-}$ mice, generated by a targeted deletion of exons 2 and 3 of the gene that blocks *Sirt6* transcription and yields no SIRT6 protein, indicated that ~60%

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[3] The abbreviations used are: AP-1, activator protein-1; SIRT, sirtuin; PCNA, proliferating cell nuclear antigen.

of homozygous mice died before 4 weeks of age when they were in a mixed genetic background of 129/Black Swiss/FVB (28). Analysis of these mutant mice demonstrated that Sirt6 negatively regulates AKT phosphorylation at Ser-473 and Thr-308 through inhibition of multiple upstream molecules, including insulin receptor, IRS1, and IRS2. The absence of Sirt6, consequently, enhances insulin/Akt/Glut1-mediated glucose uptake, leading to extreme hypoglycemia. Consistent with this hypothesis, when fed with water containing 10% glucose, the blood glucose level of the mutant mice was significantly increased, and up to 83% of these mice survived beyond the first month after birth. Of note, the majority of these mice, despite their normal levels of blood glucose, still gradually died within 1 year (28), suggesting that Sirt6 is essential for the adulthood development and survival. Consistent with this data, neural Sirt6 disruption attenuated somatic growth and caused obesity (29), whereas hepatic specific deletion of Sirt6 resulted in fatty liver formation caused by enhanced glycolysis and triglyceride synthesis (30). Conversely, transgenic mice overexpressing Sirt6 have a significantly longer lifespan than wild type mice that is accompanied by reduced insulin-like growth factor 1 signaling (31).

Recent studies have indicated that SIRT6 positively regulates TNF-α (32) and negatively regulates NF-κB signaling (33), although whether or not SIRT6 plays a role in inflammation is unknown. Moreover, because both TNF-α and NF-κB are potent proinflammatory cytokines, positively regulating one gene while negatively regulating the other generates contradictory information and makes it difficult to predict a role of SIRT6 in inflammation. To decipher the functions of Sirt6 in the inflammatory response, organism survival, and lifespan, we analyzed Sirt6 mutant mice that survived post-weaning lethality. Our data indicate that these animals exhibit massive inflammation in several organs, most severely in the liver. We further demonstrate that the absence of Sirt6 results in activation of c-JUN-dependent transcription and enhanced expression of proinflammatory genes in immune cells, leading to chronic inflammation and fibrosis in the liver of mutant mice.

EXPERIMENTAL PROCEDURES

RT-PCR and Real Time PCR—Total RNA from cells or tissues was extracted with RNA STAT-60™ following the manufacturer's protocol (Tel-Test, Inc.), and cDNA was generated by Cells-to-cDNA™ II (Ambion, Inc.). Quantitative RT-PCR was performed using a SYBR green PCR Master Mix (Applied Biosystems) and the 7500 Real Time PCR system (Applied Biosystems).

Western Blot Analysis and Immunoprecipitation Assay—Western blot analysis was carried out according to standard procedures using ECL detection from Millipore (Billerica, MA). Antibody against c-JUN was from Cell Signaling Technology, and β-actin was from Sigma. For immunoprecipitation, 293T cells were transfected with SIRT6-FLAG and c-JUN-HA. FLAG M2-agarose beads (Sigma) were added to 1 mg of protein lysate for 16 h at 4 °C while rotating. The immunoprecipitates were washed three times with cell lysis buffer and resolved by SDS-PAGE on 4–12% Tris-glycine gels (Invitrogen). Antibodies against SIRT6, FLAG-M2, and HA-7 were from Sigma.

Histology, Immunohistochemistry, and Immunofluorescence Analyses—Liver tissues were fixed in 10% formalin, blocked in paraffin wax, and sectioned. For histology, the sections were stained with hematoxylin and eosin and examined by light microscopy. For immunohistochemistry and immunofluorescence, paraffin sections of 5-μm thickness were deparaffinized and hydrated through a graded alcohol series, and antigen retrieval was carried out in pressure cooker with citrate buffer, pH 6.0 for 20 min. Detection of primary antibodies was performed using the ZYMED Histomouse SP kit (Zymed Laboratories Inc. South San Francisco, CA) according to the manufacturer's instructions. Antibodies against F4/80 was from Novus Biologicals (Littleton, CO), myeloperoxidase (MPO) was from Biocare Medical (Concord, CA), CD3 was from Dako, IL-1β was from R&D, Ki-67 was from Novocastra, and PCNA was from Abcam. For CD3 and PCNA detection, after primary antibody reaction, sections were stained with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) (CD3) or Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) (PCNA) for 1 h at room temperature, washed, and mounted on slides with Prolong Gold anti-fade reagent (Invitrogen) and photographed.

ChIP Assay—ChIP assays were performed as described previously (34). The antibodies against Ac-H3K9 and Me-H3K4 were purchased from Millipore. Antibody against c-JUN was from Cell Signaling Technology, and antibody against SIRT6 was from Sigma.

RNA Interference—The sh-c-JUN lentiviral construct was purchased from Sigma (construct TRCN0000360511). The packaging and envelope vectors psPAX2 and VSV-G were obtained from Addgene. 293T cells were transfected with sh-c-JUN or sh-Luci, psPAX2, and VSV-G using FuGENE 6 for 24 h. The medium was changed and collected after 24 h, respectively. Lentiviruses were collected and used to infect the Sirt6 MT and WT macrophages. Twenty-four hours later, the cells were harvested for assessing the mRNA levels and the c-JUN protein level.

In Silico Analysis—The *in silico* analysis of promoter was performed by using the software MatInspector from Genomatix Software Gmbh.

Immortalization of Macrophages—Immortalized macrophage cell lines were established by infecting primary bone marrow cells from 3-week-old WT and *Sirt6* KO mice with the J2 recombinant retrovirus as described (35). The packaging cell line CREJ2 was adapted from the parental J2 packaging line. CREJ2 contains the murine retroviral ecotropic coat protein, and the virus produced is replication defective. Single cell suspensions from bone marrow were prepared, placed in Dulbecco's modified Eagle's medium, and centrifuged through a lymphocyte separation medium cushion (ICN Biochemicals, Aurora, OH). The cells were cocultured with 0.45-micron-filtered CREJ2 supernatants in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, 2 mM L-glutamine (complete medium) (BioSource International, Camarillo, CA), 5 mg/ml hexadimethrine bromide (Sigma) and 1000 units/ml granulocyte macrophage-colony stimulating factor (PeproTech Inc., Rocky Hill, NJ) for 24 h. Nonadherent cells were removed from cultures, and adherent cells were cultured in complete medium with 1000

units/ml granulocyte macrophage-colony stimulating factor and without hexadimethrine bromide. After 5–7 days, the cells were cultured in complete medium without granulocyte macrophage-colony stimulating factor and monitored for growth. Cells growing in the absence of granulocyte macrophage-colony stimulating factor were considered immortalized.

Kupffer Cell Isolation from Mouse Liver and Stimulation with LPS—Kupffer cells were isolated as previously described (36). Isolated Kupffer cells were treated with or without LPS (100 ng/ml). Cell supernatant was collected at 4 h post-treatment and kept at −80 °C until the levels of the inflammatory cytokines were determined.

Cytokine Assays—Inflammatory cytokines were measured using cytometry bead arrays (BD Biosciences, San Jose, CA) according to the manufacturer's protocol.

Mice—Mice carrying a $Sirt6^{\Delta 2-3}$ allele and $Sirt6^{Co}$ allele were genotyped by PCR using conditions described previously (28, 30). Mice carrying a $Sirt6^{neo}$ allele were genotyped using primers F (5′-gggacttgccctgtagatca-3′) and R1 (5′-atcgccttctatcgccttcttgacgagttc-3′); WT allele was genotyped using F and R2 (5′-gtccctgcagaagaagatgc-3′). All three lines of cre mice, Alb-Cre (37), Lck-Cre, and Lyz-Cre (Provided by Jackson laboratory), were genotyped using primer pair Cre-1 (5′-atgcttctgtccgtttgccg-3′) and Cre-3 (5′-cctgttttgcacgttcaccg-3′). All of the experiments were approved by the Animal Care and Use Committee of the National Institute of Diabetes, Digestive, and Kidney Diseases.

Statistical Analyses—Student's *t* test was used to compare differences between samples analyzed. Any *p* value of <0.05 ($p < 0.05$) was considered to be a statistically significant difference.

RESULTS

$Sirt6^{-/-}$ Mice Exhibited Chronic Inflammation in the Liver—To further study the function of Sirt6, we analyzed $Sirt6^{-/-}$ mice at varying ages in comparison with wild type control mice. We found that the mutant mice suffered progressively massive inflammation in the liver starting from 2 months of age. Upon dissection, 14 of 28 Sirt6 mutant mice examined between 2 and 8 months of age showed white foci on the surface of the liver (Fig. 1*A* and Table 1). As the mutant animals were getting older, the frequency of surface white foci was reduced; however, many animals developed visible nodules and rough surface on the liver (Fig. 1, *B–D*, and Table 1). Hematoxylin and eosin analysis on sections cutting through the white foci revealed patches of necrotic cells that were surrounded by leukocytes (Fig. 1*F*). Many of these necrotic areas were embedded inside the liver, without displaying white foci on the surface. Leukocytes were found throughout the entire liver of these mice (Fig. 1*G*). These abnormalities were not observed in control mice (Fig. 1, *E* and *H*).

Our further analysis indicated that the majority of the infiltrated inflammatory cells were CD3 positive (Fig. 1*I*). A considerable number of inflammatory cells were also positively stained by markers for macrophage (F4/80) (Fig. 1*J*) and neutrophil (MPO) (Fig. 1*K*). High levels of IL-1β, a cytokine that is usually secreted by inflammatory cells, was also detected in the $Sirt6^{-/-}$ liver (Fig. 1*L*), whereas the WT controls showed much less staining for these markers (Fig. 1, *N–P*). Consistent with these phenotypes, we detected increased transcription of several proinflammatory genes in *Sirt6* mutant mouse livers, including monocyte chemotactic protein 1 (*Mcp-1*), *Il-6*, chemokine C-C motif ligand 5 (*Ccl5*), *Il-1β*, cyclin D1, vascular cell adhesion molecule 1 (*Vcam*), *Ifn-γ*, *Tnf-α*, and inducible nitric oxide synthase (*iNos*), as well as C-C chemokine receptor type (*Ccr2*), marker for monocytes, and *F4/80*, marker for Kupffer cells/macrophages (Fig. 1*Q*). We also detected variable levels of inflammation in several other organs, including kidney, pancreas, and lung, which were much less severe compared with those in the liver (supplemental Fig. S1).

We have examined $Sirt6^{-/-}$ mice ($n = 6$) at age of 9 months and detected elevated serum alanine aminotransferase and aspartate aminotransferase compared with wild type mice; however, the increase did not reach a statistically significant level (supplemental Fig. S2*A*), suggesting the mutant mice might suffer a slightly or moderately impaired liver function at this age. *Sirt6* mutant mice at this age also exhibited moderate yet nonstatistically higher serum levels of IL-6, MCP-1, and TNFα than did the wild type mice (supplemental Fig. S2*B*). Next, we examine liver fibrosis by performing Sirius Red staining on the $Sirt6^{-/-}$ mouse liver. Our data revealed that significant fibrosis was detected in the $Sirt6^{-/-}$ livers but not in wild type mouse livers (Fig. 2, *A* and *B*). We also detected some hypercellular areas containing proliferation cell nuclear antigen-positive ($PCNA^{+}$) hepatocytes (Fig. 2*C*, *white arrow*), suggesting increased cellular proliferation. Double staining using antibodies for PCNA and CD3 indicated that some $PCNA^{+}$ cells are CD3 positive (membrane staining, *arrowhead*), indicating that some leukocytes were also proliferative. The staining of these two types of cells only appeared in Sirt6 MT (Fig. 2*C*), but not in WT (Fig. 2*D*) livers. Of note, both antibodies for PCNA and CD3 also stained nonspecifically for red blood cells, which have no nucleus (*red arrow*) in both Sirt6 MT (Fig. 2*C*) and WT (Fig. 2*D*) livers. As the liver abnormalities were getting more severe in older animals, we also detected steadily decreased body weight in the older Sirt6 mutant mice (Fig. 2*D*). We also detected higher liver weight/body weight ratio in the *Sirt6* mutant mice at 4–6 month of age compared with WT mice (Fig. 2*E*), although no significant changes were detected in other age groups. Altogether, these data indicated that Sirt6 mutant mice developed severe liver inflammation, liver fibrosis, and declining health, conditions that might contribute to the lethality that was reported earlier (28).

Liver Inflammation Is Primarily Caused by Sirt6 Deficiency in the Lymphocytes and Myeloid-derived Cells Rather than in the Hepatocytes—Because the inflammation occurred primarily in the liver, we investigated whether the absence of Sirt6 in hepatocytes is a primary cause for the inflammation. We first examined a mutant strain carrying hepatocyte-specific mutation of Sirt6 ($Sirt6^{Co/Co}$;*Alb-Cre*) (30) and detected no obvious inflammation in these mice, suggesting that Sirt6 deficiency in the hepatocytes alone does not trigger inflammation.

Next we deleted the *pLoxPneo* gene from the $Sirt6^{Neo/Neo}$ mice using *Alb-Cre* to restore Sirt6 expression specifically in hepatocytes (supplemental Fig. S3, *A* and *B*). Our examination of the $Sirt6^{Neo/Neo}$;*Alb-Cre* mice detected a similar inflamma-

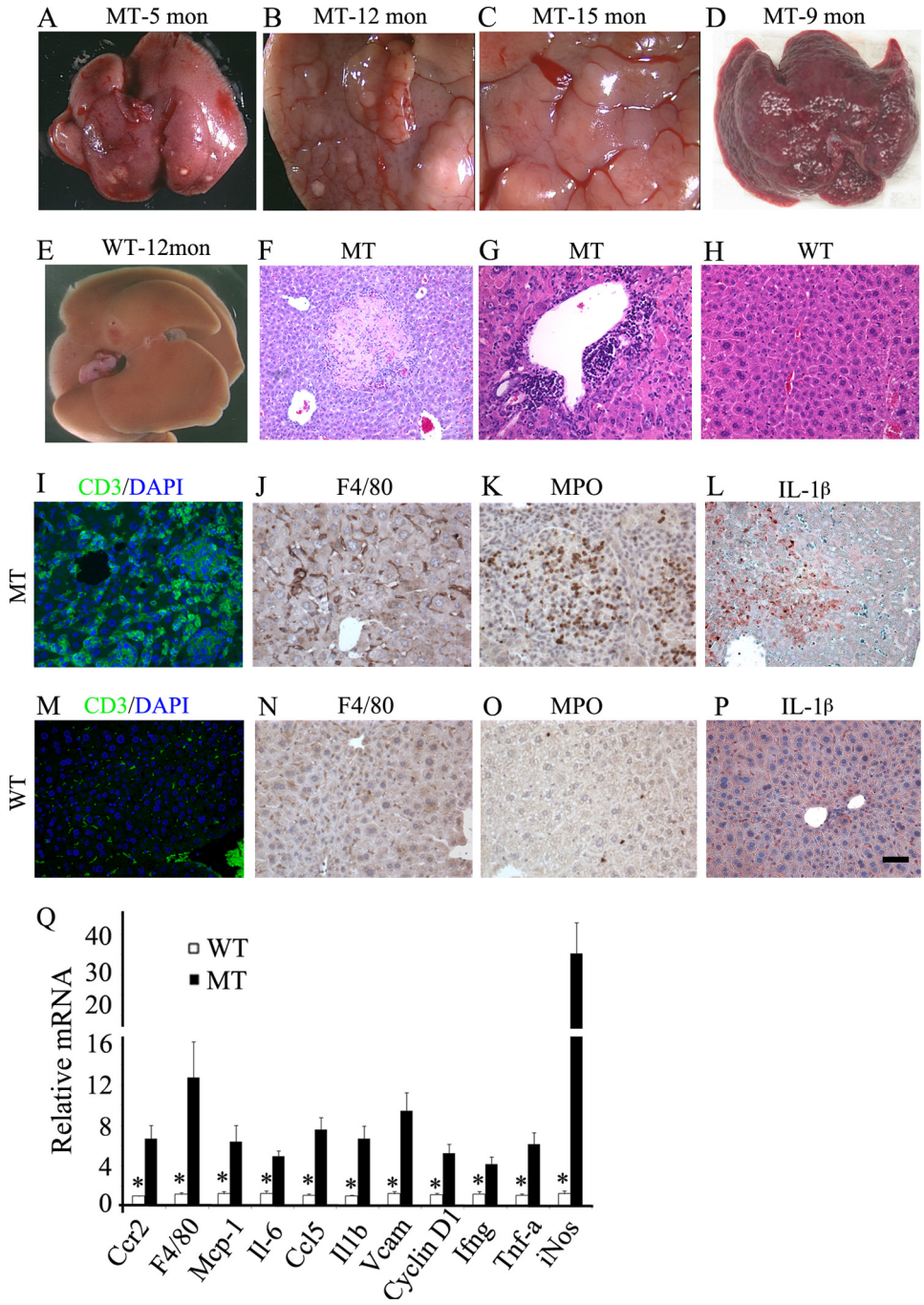

FIGURE 1. **Liver inflammation in Sirt6 mutant mice.** *A–E*, gross morphological changes of Sirt6 mutant (*MT*) (*A-D*) and WT (*E*) liver. Liver foci (*A*) in a 5-month-old animal and nodules (*B–D*) in 9–15-month-old animals. *F–H*, hematoxylin and eosin staining showing necrotic foci surrounded by inflammatory cells (*F*) and massive inflammation (*G*) in MT and WT (*H*) liver sections. *I* and *M*, CD3 staining in MT (*I*) and WT (*M*) liver section. The *green signals* in *M* are nonspecific staining of red blood cells. *J–L* and *N–P*, F4/80, MPO, and IL-1β immunohistochemistry staining in Sirt6 MT (*J–L*) and WT (*N–P*) liver. *Q*, hepatic expression of proinflammatory genes. The *error bars* indicate standard error of the mean. *, $p < 0.05$. *Scale bar*, 100 μm (*F*); 50 μm (*G–P*). For *A–P*, at least five pairs of mice were analyzed. For *Q*, three pairs of 8–9-month-old mice were analyzed.

tory phenotype in the liver compared with the *Sirt6*$^{-/-}$ liver described earlier (supplemental Fig. S3, *C* and *D*). These data indicate that the inflammation observed in the *Sirt6*$^{-/-}$ mice was due to *Sirt6* deficiency in the cell types other than hepatocytes.

Because T cells and macrophages are predominant immune cells in the inflammatory liver in the absence of Sirt6, we generated two additional mutant strains carrying the T cell-specific (*Sirt6*$^{Co/Co}$*;Lck-Cre*) and the myeloid-derived cell-specific (*Sirt6*$^{Co/Co}$*;Lyz-Cre*) knock-out of Sirt6, respectively. We revealed that both lines of mice showed liver inflammation, with the latter showing more severe phenotypes than the former, although both of them showed delayed onset of inflammation compared with *Sirt6*$^{-/-}$ mice (supplemental Table S1). Both lines of mice also showed some degrees of liver fibrosis (data not shown). These data suggest that the inflammation in *Sirt6*$^{-/-}$ mice is caused by Sirt6 deficiency in more than one type of immune cells, and the deletion of Sirt6 in myeloid-de-

TABLE 1
Liver inflammation in Sirt6 KO mice

Genotype	Age	No. of mice	Liver inflammation
	months		
WT	2–18	30	Four have moderate inflammation, and no white foci, necrotic foci, and nodules were observed.
KO	2	5	All had liver inflammation revealed by hematoxylin and eosin, and three had white foci on the liver surface.
	3–4	4	Three had liver inflammation, two had white foci on the liver surface, and two showed necrotic foci on liver sections.
	5–6	10	Nine had liver inflammation, two had white foci on the liver surface, and six showed necrotic foci on liver sections.
	7–8	9	Nine had liver inflammation, seven had white foci on the liver surface, and one had rough liver surface.
	9–10	6	All had liver inflammation, one had white foci on the liver surface, and three had rough liver surface and small nodules.
	11–12	9	Eight had liver inflammation, one had white foci on the liver surface, and one had lots of small nodules.
	>12	3	Three had liver inflammation, and one had lots of small nodules on the ventricular side.

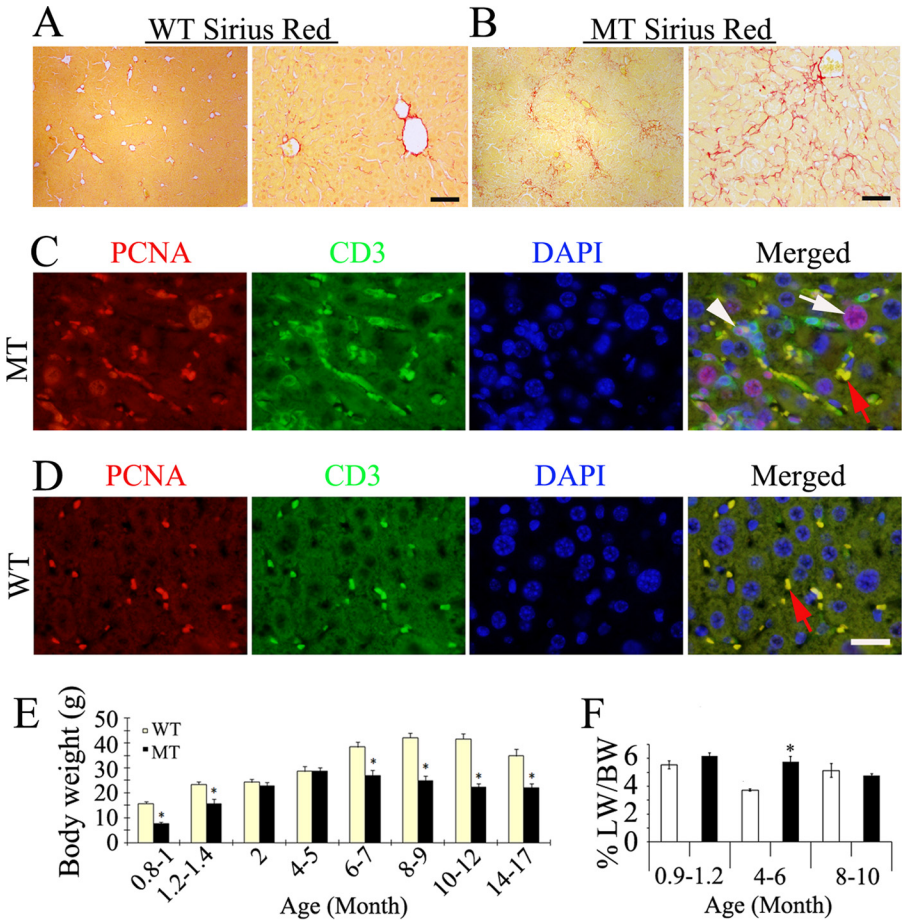

FIGURE 2. **Sirt6 loss results in liver fibrosis, elevated liver proliferation, and global decline of body condition.** *A* and *B*, Sirius Red staining images in WT (*A*) and mutant (*MT*) (*B*) mice liver. Eight pairs of mice were analyzed. *C* and *D*, PCNA and CD3 staining in MT (*C*) and WT liver (*D*). The *white arrow* indicates PCNA positive only cell (most likely hepatocyte according to the morphology). The *arrowhead* indicates PCNA and CD3 double positive cell (leukocyte). The *red arrows* indicate nonspecific staining of the red blood cells. At least five pairs of mice were analyzed for *C* and *D*. *Scale bars*, *A* and *B*, *left panel*, 250 μm; *right panel*, 50 μm. *C* and *D*, 20 μm. *E*, body weight of WT and MT mice. *F*, percentage of liver weight (*LW*) *versus* body weight (*BW*) in three time points of WT and MT mice. The *error bars* indicate the standard error of the mean. *, $p < 0.05$. At least four pairs of mice were analyzed for *E* and *F*.

rived cells correlated with a more severe phenotype than in T cells.

Sirt6 Loss Activates T Cells and Myeloid-derived Cells Leading to Liver Inflammation—To understand how Sirt6 deficiency in T cells and monocytes causes liver inflammation, we isolated these cells from the liver and performed flow cytometry analysis using various antibodies. Our data showed that *Sirt6*$^{-/-}$ liver contained a higher frequency of CD4$^+$ T cells compared with those in wild type mice (Fig. 3*A*). It has been known that CD4$^+$ T cells can induce contact-independent target cell death through secreting a number of soluble molecules, including IFN-γ (38). Consistent with this, we observed an increased proportion of IFN-γ secreting CD4$^+$ T cells from *Sirt6*$^{-/-}$ liver (Fig. 3*A*). It was reported that macrophages and CD4$^+$ T cells often cooperate to trigger a pronounced inflammatory reaction (39, 40). To investigate this, we isolated Kupffer cells and liver resident macrophages and found that *Sirt6*$^{-/-}$ Kupffer cells had elevated levels of IL-6 and TNFα under LPS stimulation and elevated basal TNFα level when compared with cells isolated from WT controls (Fig. 3*B*). The increased production of IFN-γ, IL-6, and TNFα produced by CD4$^+$ T cells and Kupffer cells in the mutant liver likely serves as a main cause of the liver inflammatory response observed in these mice.

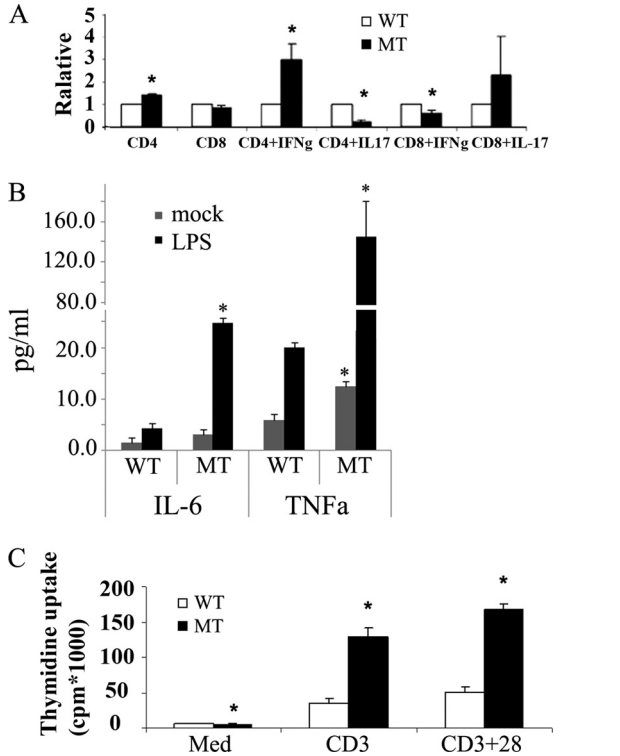

FIGURE 3. **Sirt6 loss activates immune cells leading to the liver inflammation.** *A*, $CD4^+$, $CD8^+$, IFN-γ, and IL-17 secretion in $CD4^+$ liver lymphocytes. *B*, IL-6 and TNFα secretion from Kupffer cells isolated from mouse liver. *C*, thymidine uptake of splenocytes under the stimulation of anti-CD3 alone and anti-CD3 + CD28. *MT*, mutant.

The increase in IFN-γ^+CD4^+ cells in *Sirt6*$^{-/-}$ liver indicated that Sirt6 regulates T cell activation. To investigate this, we cultured splenocytes with or without anti-CD3 and/or soluble anti-CD28 for 3.5 days and revealed that *Sirt6*$^{-/-}$ T cells showed a significantly higher proliferation rate than did WT splenocytes upon T cell receptor stimulation (Fig. 3*C*). These data indicate that immune cells in *Sirt6* mutant mice are activated even before they migrate to the liver. TGF-β signaling plays an important role in triggering an inflammatory response (4). However, our analysis indicated that both *Sirt6*$^{-/-}$ and WT splenocytes exhibited a similar response to TGF-β treatment (data not shown), suggesting that Sirt6 loss does not have an obvious effect on the responsiveness of TGF-β signaling.

Sirt6 Deficiency Enhances Inflammatory Cytokine Production in Macrophages—To understand the underlying mechanism responsible for the activation of the immune response in *Sirt6*$^{-/-}$ mice, we established macrophage cell lines from bone marrow isolated from the *Sirt6*$^{-/-}$ and WT mice and analyzed the cytokine production by these cells. Under basal conditions, IL-6, MCP-1, and TNFα levels are significantly higher in the *Sirt6*$^{-/-}$ macrophage than those in the WT macrophage (Fig. 4, *A* and *B*), suggesting that *Sirt6*$^{-/-}$ cells consistently make more of these cytokines. Upon LPS stimulation, production of these cytokine markedly increased in both types of cells (Fig. 4, *A* and *B*), suggesting that Sirt6 is not required for the inducible expression of these cytokines, although our data indicate that *Sirt6*$^{-/-}$ cells still produced significantly more MCP-1 than WT cells (Fig. 4*B*). Consistent with the changes in IL-6 and MCP-1 production, we also observed a significant increase of the *Il-6* and *Mcp-1* mRNA levels in the *Sirt6*$^{-/-}$ cells (Fig. 4, *C* and *D*). The LPS-induced up-regulation of *Il-6* and *Mcp-1* mRNAs requires gene transcription because actinomycin D treatment completely blocked transcription of these genes (Fig. 4, *E* and *F*). In addition, we also observed significantly increased mRNA levels of *Il-1β*, *Ccr2*, *Tnf-α*, *iNos*, and *F4/80* (Fig. 4, *G–K*) in the *Sirt6*$^{-/-}$ macrophage cell line.

Sirt6 Deacetylase Epigenetically Modifies Histone in the Promoter of Proinflammatory Genes—To investigate the potential mechanisms of how Sirt6 deficiency increases expression of proinflammatory genes, we analyzed their promoters. Using an *in silico* analysis, we detected evolutionarily conserved binding sites for activator protein 1 (AP-1) or AP-1-related transcription factor in the promoter of both the human and mouse *IL-6* and *MCP-1* genes (Fig. 5, *A* and *B*). Previous studies also indicated that Sirt6 negatively regulates gene expression through deacetylating histone H3 lysine 9 (H3K9) in the promoter of its target genes (30, 33). To investigate this possibility, we performed ChIP assay of AcH3K9 in the *Il-6* and *Mcp-1* promoters using the *Sirt6*$^{-/-}$ and WT macrophage cell lines. Our data indicated that in the *Sirt6*$^{-/-}$ macrophages, AcH3K9 levels on both *Il-6* and *Mcp-1* promoters dramatically increased in comparison with the WT macrophages (Fig. 5*C*). A high level of AcH3K9 indicates an open chromatin configuration. Consistently, we detected increased levels of two additional histone markers for open chromatin configuration, Me2H3K4 and Me3H3K4, in the promoter of *Il-6* gene (Fig. 5, *D* and *E*). However, such an increase was not observed in the *Mcp-1* promoter (Fig. 5, *D* and *E*); perhaps these two modifications do not play an obvious role in the promoter of the *Mcp-1* gene, because different promoters may be regulated differently. To investigate this further, we analyzed whether SIRT6 binds to these regions on the promoters of both the *Il-6* and *Mcp-1* genes. We detected more obvious occupancy of SIRT6 in the promoter of both genes in wild type cells in comparison with *Sirt6*$^{-/-}$ cells (Fig. 5*F*). These data indicate that SIRT6 binds and deacetylates H3K9 in the promoters of these proinflammatory genes.

SIRT6 Interacts with c-JUN and Negatively Regulates Inflammation Signaling—AP-1 is a homodimer or heterodimer protein composed of proteins belonging to the c-FOS, c-JUN, activating transcription factor, and musculoaponeurotic brosarcoma protein families (3, 41). Activation of c-JUN/AP-1 and increased expression of their downstream proinflammatory genes, including IL-6 and MCP-1, have been found to cause inflammation in several tissues, including skin, lung, and bone (3, 42, 43). Because SIRT6 binds to and deacetylates H3K9 in the promoter of these genes and Sirt6 deficiency results in their up-regulation, we hypothesized that SIRT6 and c-JUN might work together to repress the transcription of these genes. To test this hypothesis, we checked their protein interaction, and the data demonstrated that SIRT6 and c-JUN indeed interact with each other (Fig. 5*G*). Of note, we also detected remarkably increased levels of c-JUN occupancy in the promoter of these genes in *Sirt6*$^{-/-}$ cells (Fig. 5*H*). These data suggest that increased expression of these proinflammatory genes could be a result of enhanced transcriptional activity of c-JUN upon *Sirt6* deficiency. If this is the case, we hypothesized that inhibition of

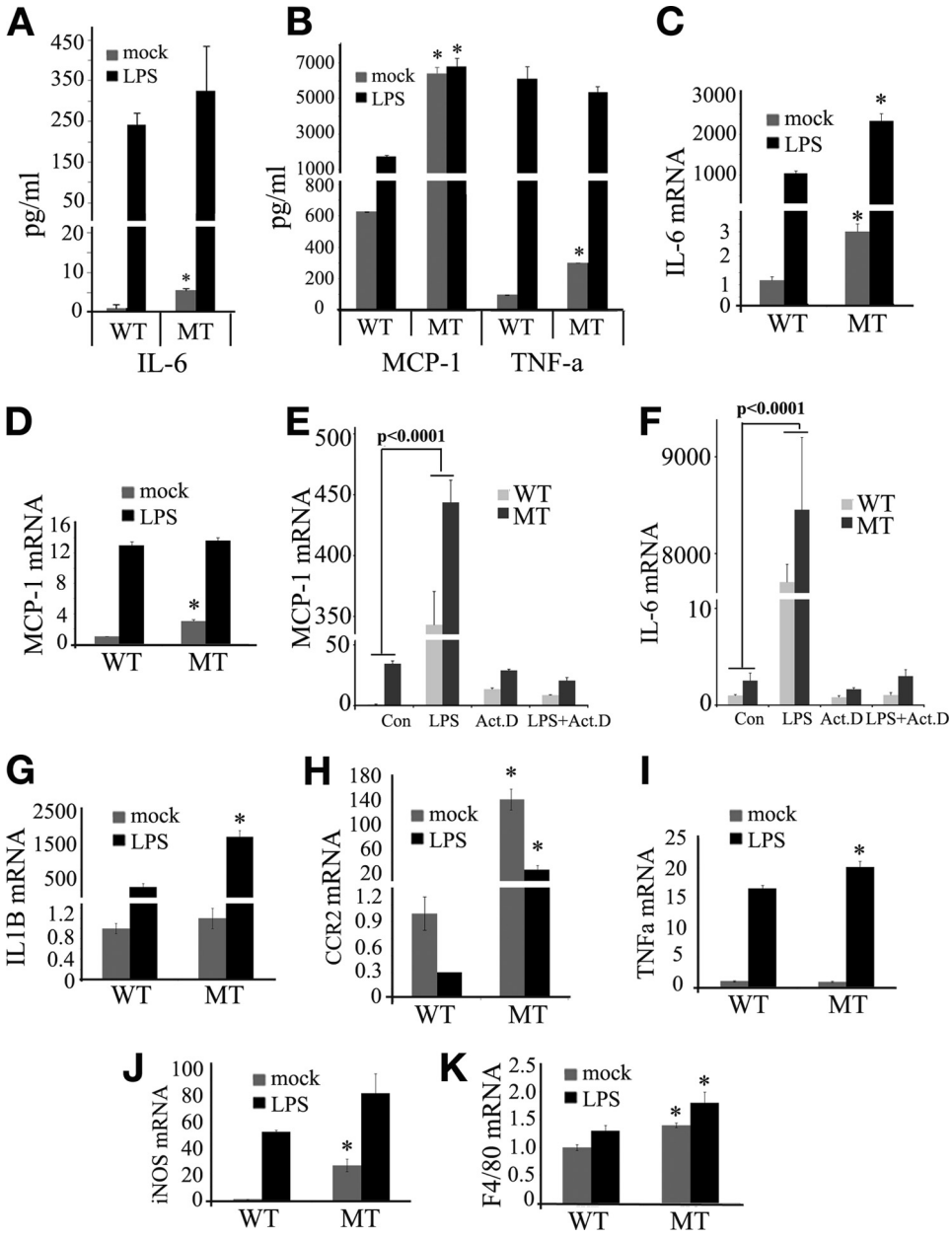

FIGURE 4. **Activation of *Sirt6* mutant macrophages.** *A* and *B*, cytokines secreted from the macrophage cell lines with and without LPS stimulation. *C–K*, proinflammatory gene expression in the macrophage cell lines. *E* and *F*, levels of LPS-induced up-regulation of *Il-6* and *Mcp-1* mRNAs with or without actinomycin D. The *error bars* indicate standard error of the mean. *, $p < 0.05$. *MT*, mutant.

c-JUN should be able to reverse the increased expression of these genes. To test this, we performed *c-JUN* knockdown in the macrophage cell line and found that *Il-6* and *Mcp-1* mRNA levels in the *Sirt6* deficient cells were brought back to similar levels as observed in the WT cells (Fig. 5*I*). These data strongly support the model that the highly activated inflammatory response in the *Sirt6*$^{-/-}$ macrophages was due to activated c-JUN signaling.

DISCUSSION

In this study, we revealed that *Sirt6*$^{-/-}$ mice developed invariably chronic liver inflammation starting at ~2 months of age. Older mutant mice also developed liver fibrosis and some hypercellular areas with increased cellular proliferation. Further analysis indicated that the liver inflammation and fibrosis are mainly caused by the activation of proinflammatory signaling in multiple types of immune cells, and we uncovered a novel role for Sirt6 in inhibiting inflammation through repressing c-JUN/AP-1 signaling.

The *Sirt6*$^{-/-}$ mice carry a global disruption of Sirt6. Why do they exhibit much more severe inflammation in the liver than other tissues? The liver is an organ that plays an essential role in removing pathogens, antigens, and viruses entering from the circulation. Upon infection or injury, leukocytes are recruited to the liver through hepatic sinusoids, which are low flow vascular channels (1, 44). The accumulation of leukocytes and their slow clearance, in addition to the large quantity of Kupffer cells, may serve as a source for initiation of inflammation and

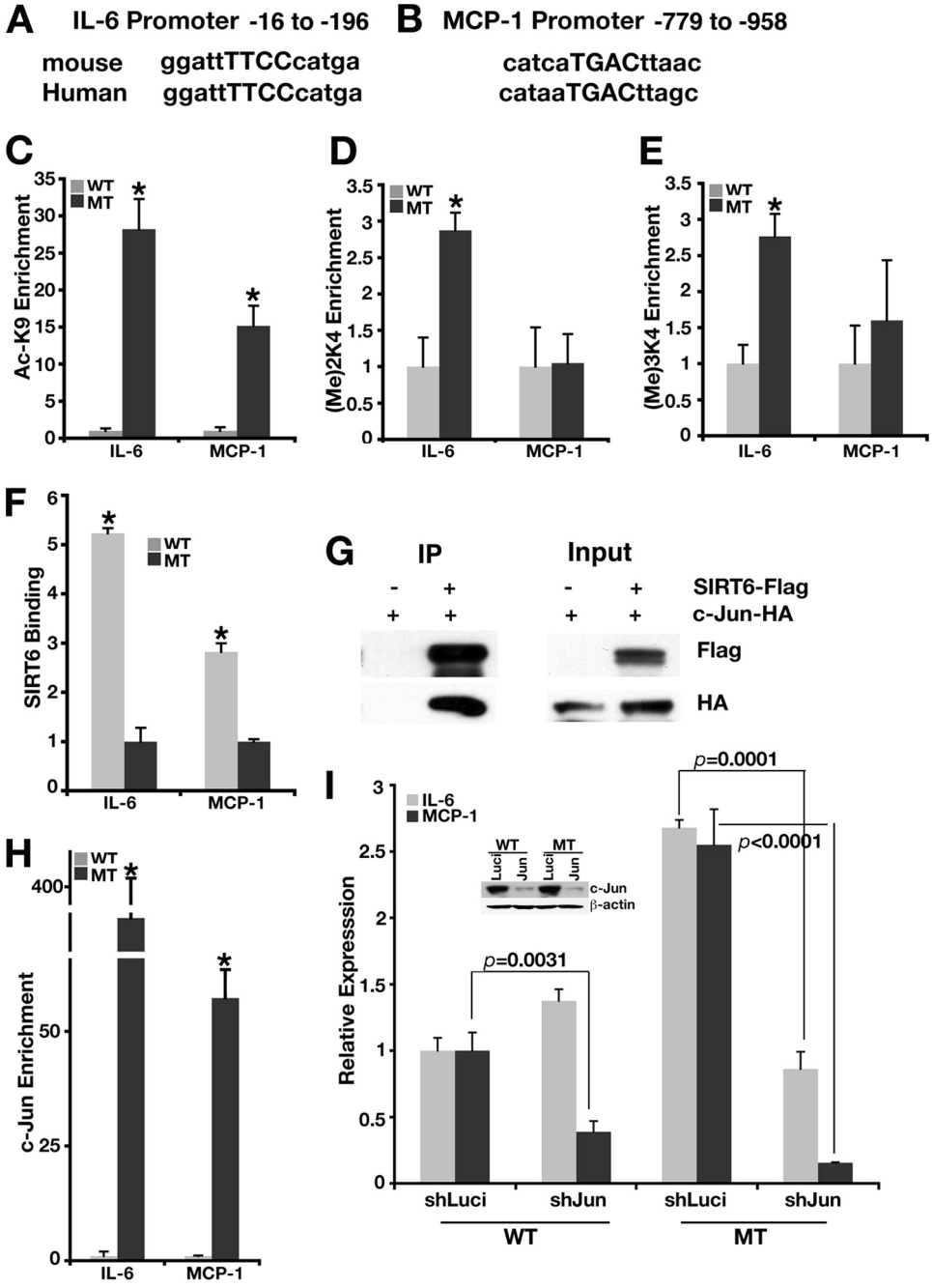

FIGURE 5. **SIRT6 interacts with c-JUN and binds to and epigenetically modifies H3K9 in the promoter of proinflammatory genes.** Immortalized WT or MT macrophages were utilized for ChIP analysis. *A* and *B*, *in silico* analysis to show AP-1 or AP-1-related binding sites on IL-6 and MCP-1 promoters from both mouse and human genes. The *numbers* indicate the positions of the fragments relative to translational start codon (ATG), which were amplified by PCR during ChIP assay. *C*, ChIP assay showing enhanced Ac-H3K9 occupancy on *Il-6* and *Mcp-1* promoters upon *Sirt6* deletion. *D* and *E*, ChIP assay showing Me-H3K4 occupancy on *Il-6* and *Mcp-1* promoters. *F*, ChIP assay indicates SIRT6 binds to *Il-6* and *Mcp1* promoters in wild type cells. The background binding levels in mutant cells were set at 1. *G*, immunoprecipitation assay displays that c-JUN and SIRT6 interact with each other. *H*, ChIP assay using c-JUN antibody demonstrates that upon *Sirt6* deletion, the binding of c-JUN to *Il-6* and *Mcp-1* promoters was extremely enhanced. *I*, expression of *Il-6* and *Mcp-1* upon *c-JUN* knockdown in both WT and MT immortalized macrophages. The *inset* indicates Western blot of c-JUN upon *c-JUN* knockdown.

fibrosis. In this study, we found that *Sirt6* deficiency activates multiple types of leukocytes, including T cells, Kupffer cells, and monocytes in other organs leading to widespread inflammation. However, the inflammation is most pronounced in the liver. To reinforce this model, we have performed the following three experiments: 1) specific disruption of Sirt6 in hepatocytes, 2) restoration of Sirt6 transcription in the hepatocytes specifically in *Sirt6*$^{-/-}$ mice, and 3) deletion of Sirt6 in T cells or myeloid-derived cells. All of these experiments came up with the same conclusion that Sirt6 deficiency in the immune system causes liver inflammation and fibrosis, whereas a loss of Sirt6 in the hepatocytes alone does not result in inflammation.

In theory, liver inflammation can be initiated by abnormalities in hepatocytes, leukocytes, or the combination of both (45). For example, targeted disruption of the tumor suppressor gene, cylindromatosis (CYLD), specifically in hepatocytes resulted in

liver inflammation, fibrosis, and cancer (46). In this case CYLD deficiency triggers hepatocyte death via spontaneous and chronic activation of TGF-β-activated kinase 1 and JNK. There is subsequent compensatory proliferation leading to tumorigenesis when the animals reached 1 year of age. In contrast, *Sirt6*$^{-/-}$ mutant liver does not exhibit obvious apoptosis revealed by TUNEL assay (data not shown) and does not have statistically significant elevation of serum alanine aminotransferase and aspartate aminotransferase. This suggests that *Sirt6* deficiency in hepatocytes does not cause strong liver injury. These observations may explain why a loss of Sirt6 in hepatocytes alone does not cause inflammation. We have also examined 12 *Sirt6*$^{-/-}$ mice between 11 and 17 months of age and did not detect cancer formation in the liver and other organs. Thus, it is unlikely that Sirt6 has a tumor suppressor function in mice. Although recent studies have indicated that Sirt1 (24, 47), Sirt2 (48), and Sirt3 (49, 50) may suppress tumorigenesis in mice and Sirt7 may promote it (51), it is conceivable that Sirt6 plays a distinct role in this process.

The most notable finding in this study is that Sirt6 deacetylase plays an essential role in mediating the inflammatory response through inhibiting c-JUN signaling. Both c-JUN and its activator, JNK, play an important role in many biological processes, including apoptosis, cell differentiation and proliferation, metabolism, and tumorigenesis in the liver (52). We showed here that SIRT6 binds to the promoters of both IL-6 and MCP-1 and deacetylates H3K9. A loss of Sirt6 results in enhanced expression of these genes, suggesting that Sirt6 negatively regulates their expression. Our data clearly demonstrate that the loss of Sirt6 enhanced c-JUN occupancy in the promoters of these genes, and more importantly, shRNA-mediated acute knockdown of c-JUN reversed activated transcription of these genes in *Sirt6* mutant cells. These observations provide strong evidence that *Sirt6* deficiency enhances *Il-6* and *Mcp1* gene expression through activation of c-JUN transcriptional activity.

A previous study indicated that SIRT6 interacts with the NF-κB RelA subunit and deacetylates H3K9 at NF-κB target gene promoters, the loss of Sirt6 caused activation of NF-κB dependent gene expression, and haploinsufficiency of RelA rescues the early lethality and degenerative syndrome of *Sirt6* deficient mice (33). However, it was also reported that overexpression of wild type or a catalytically dead mutant of SIRT6 do not influence NF-κB responses (53). Because activation of NF-κB is frequently observed in chronic liver inflammation (3), we have generated and analyzed *Sirt6*$^{-/-}$;*p65*$^{+/-}$ mice. We did not find an obvious difference in phenotypes between the *Sirt6*$^{-/-}$;*p65*$^{+/-}$ mice and *Sirt6*$^{-/-}$ mice, suggesting that haploinsufficiency of RelA did not attenuate liver inflammation (data not shown). In addition, we have also performed acute knockdown of NF-κB in the *Sirt6*$^{-/-}$ macrophages and found it also caused reduction of *c-JUN* transcription (data not shown), suggesting that NF-κB and c-JUN might modulate each other, consistent with the earlier report of a similar modulation between NF-κB and c-JUN/AP-1 (54). Thus, the relationship among NF-κB, c-JUN/AP-1, and SIRT6 is complex and deserves future investigation.

In summary, previous studies have revealed that Sirt6 is involved in many important biological processes, including glucose metabolism, lipid metabolism, triglyceride synthesis, DNA damage repair, telomere maintenance, and lifespan regulation (17, 28, 30, 31, 55, 56). Our study uncovers a novel anti-inflammatory role of Sirt6 through inhibiting c-JUN signaling. This finding may have a significant clinic application in the future because chronic inflammation causes many diseases including fatty liver diseases, cirrhosis, and hepatocarcinoma (5–8). In this case, small chemical compounds that activate Sirt6 deacetylase should be useful in therapeutic treatment to decrease inflammation and improve patient symptoms.

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